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ORIGINAL ARTICLE

Analytical quality-by-design approach for sample treatment of BSA-containing solutions

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KEYWORDS

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Analytical quality-by-design (QbD);
Sample preparation;
Design of experiment (DOE);
Derringer desirability (D)

Abstract The sample preparation of samples containing bovine serum albumin (BSA), *e.g.,* as used in transdermal Franz diffusion cell (FDC) solutions, was evaluated using an analytical quality-by-design (QbD) approach. Traditional precipitation of BSA by adding an equal volume of organic solvent, often successfully used with conventional HPLC-PDA, was found insufficiently robust when novel fused-core HPLC and/or UPLC-MS methods were used. In this study, three factors (acetonitrile (%), formic acid (%) and boiling time (min)) were included in the experimental design to determine an optimal and more suitable sample treatment of BSA-containing FDC solutions. Using a QbD and Derringer desirability (*D*) approach, combining BSA loss, dilution factor and variability, we constructed an optimal working space with the edge of failure defined as $D<0.9$. The design space is modelled and is confirmed to have an ACN range of $83\pm3\%$ and FA content of $1\pm0.25\%$.

1. Introduction

Within the context of *in vitro* dermal absorption studies, the choice of receptor fluid is a very important factor with the major consideration of the receptor fluid not acting as a rate-limiting step in the permeation process due to the limited solubility of the test compound in the medium [1]. During these Franz diffusion cell (FDC) experiments, usually aqueous-based solutions are used as receiver phase beneath the mounted human or animal skin section membranes [2]. However, it is well recognized that for lipophilic compounds this may cause flux limiting solubility or unstirred layer phenomena which can obscure the true flux of the drug through the skin. In such cases, it is advised to use a solubilising additive in order to guarantee sink conditions throughout the experiment [1–2]. In almost 20% of recent studies, bovine serum albumin (BSA) was used in a

Abbreviations: ACN, acetonitrile; BSA, bovine serum albumin; D, Derringer desirability; DF, dilution factor; DOE, design of experiments; FA, formic acid; FDC, Franz diffusion cell; MLR, multiple linear regression; PBS, phosphate buffered saline; QbD, quality-by-design

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concentration up to 5% (m/V), in which it also contributed to the *in vivo* relevance [3–4].

During the FDC studies, samples of receptor fluid are taken at regular time intervals in order to calculate the cumulative concentration of permeated drug and its kinetic permeation parameters. Prior to the chromatographic analysis of the drug, a sample preparation is required to eliminate the BSA protein and liberate the drug before HPLC injection. Beside the many existing techniques for deproteinising samples, the traditional and most simple approach is to add an equal volume of organic solvent, *e.g.* acetonitrile. After centrifugation of the precipitated BSA, an aliquot of the clear supernatant is then injected into the HPLC system [3–10].

While this approach was often successful for traditional HPLC-UV analysis, recent experience within our laboratory has indicated that such a simple sample preparation is insufficient when switching to more advanced fused-core and/or UPLC-MS systems. This has led to bad peak shapes with shifted retention times and increased variability, giving unreliable results, but has also led to increased column back pressure, insufficient column robustness, which can also drastically shorten its life span.

The objective of this work was to determine an optimal and more suitable sample treatment of BSA-containing FDC solutions, aiming at a BSA precipitation of minimally 95%. We applied an analytical quality-by-design (QbD) approach, using a multivariate experimental design to construct a design space.

2. Materials and methods

2.1. Reagents

HPLC gradient grade acetonitrile (ACN) was supplied by Fisher Scientific (Leicestershire, UK). LC-MS formic acid (FA) was purchased from Fluka (Buchs, Switzerland). Water was purified using an Arium 611 purification system (Sartorius, Göttingen, Germany), resulting in ultrapure water of 18.2 MΩ cm quality. Phosphate buffered saline (PBS) (pH 7.4; 0.01 M) and Bradford reagent came from Sigma (St. Louis, MO, USA). BSA used for the FDC samples was bought from Merck (Darmstadt, Germany), while the 2 mg/mL BSA protein standard stock for analytical quantification came from Thermo Scientific (Waltham, MA, USA).

2.2. Experimental design and modelling

In this study, three factors were examined: boiling time (0.25–3 min), formic acid content (0.5–5%) and acetonitrile content (70–90%). The main and first response, *i.e.*, percentage BSA loss, was calculated according to the following formula: BSA loss (%) = (1–([BSA]$_{left}$/[BSA]$_{applied}$)) × 100%, where [BSA]$_{left}$ is the calculated BSA concentration after sample preparation and [BSA]$_{applied}$ is the

Table 1 Experimental set-up and results.

Exp. no.	Factors and levels			Dilution factor (DF)	Blue colored reaction	BSA loss (%)	RSD (%)
	Boiling time (min)	FA (%)	ACN (%)				
1	1	0.5	70	4.5	-	97.07	47.13
2	1.75	0.5	70	4.5	-	97.61	
3	1.75	2.75	70	4.5	+	37.65	
4	1.75	2.75	70	4.5	+	38.96	
5	1.75	2.75	70	4.5	+	39.40	
6	1	2.75	70	4.5	+	40.82	
7	3	2.75	70	4.5	+	41.15	
8	1	2.75	70	4.5	+	42.97	
9	0.5	2.75	70	4.5	+	45.29	
10	1	2.75	70	4.5	+	46.40	
11	0.25	2.75	70	4.5	+	46.62	
12	1.75	2.75	70	4.5	+	53.71	
13	1.75	5	70	4.5	+	11.23	
14	1	5	70	4.5	+	38.02	
15	0.25	0.5	80	5.5	-	99.04	0.15
16	1.75	0.5	80	5.5	-	99.12	
17	1	2.75	80	6	-	99.13	
18	1.75	5	80	7	-	98.78	
19	0.25	5	80	7	-	98.88	
20	3	0.5	90	19	-	96.72	0.50
21	0.5	0.5	90	19	-	97.00	
22	1.75	2.75	90	19	-	96.91	
23	3	5	90	23.5	-	95.83	
24	0.5	5	90	23.5	-	96.29	

$DF = \frac{V_{total}}{V_{sample}}$ with $V_{total} = V_{H_2O} + V_{FA} + V_{ACN} + V_{sample}$ and V_{sample} = 200µL.

theoretical BSA concentration added at the start of the experiment. Two other responses were also considered, *i.e.*, dilution factor (DF) defined as $DF = V_{total}/V_{sample}$ and the variability expressed as RSD (in percentage). From these three individual responses, a global Derringer desirability (D) was calculated.

Previous pilot experiments already indicated that relatively low ACN levels, *i.e.*, 50% and 60%, resulted in a BSA loss that was too low to be usable and characterized by an extremely high variability. Therefore, these levels were excluded from further experiments, obtaining a model containing all experimental data available in a justified region.

Multivariate data-analysis and modelling was performed using Modde 8.0.2 software (Umetrics, Umea, Sweden). The BSA loss data, expressed as percentage, as well as dilution factor (DF), variability (expressed as RSD) and Derringer desirability (D) were used as dependent variables to fit the most appropriate multiple linear regression (MLR) model and to construct contour plots.

2.3. *Sample preparation*

A well-defined volume of FA, ACN and H_2O was brought into an adequate tube according to the established design, with H_2O serving as an adjuvant to match the degrees of freedom. Next, 200 µL of a 3% BSA in PBS solution was added to each tube; this sample quantity and BSA concentration are normally used in FDC skin studies. Different volumes were thus obtained, resulting in different dilution factors of the original BSA-containing sample. All recipients were then thoroughly shaken and vortexed to ensure complete homogenization. Of each sample solution, a 400 µL aliquot was transferred to a 0.5 mL Eppendorf tube, after which the tubes were placed in a boiling water bath for a predetermined time (Table 1). Immediately thereafter, samples were placed on ice for 15 ± 5 min and centrifuged at a relative centrifugational force of 20,800 g for 10 min at room temperature. The supernatant was then subjected to the Bradford protein assay.

Table 2 Summary of model fitness for BSA loss % = –69.39 FA+42.68 ACN+0.80 FA × ACN − 0.27 ACN^2–1601.55.

Performance indicators	Reference values [14]	Results of our model	
R^2	~1	0.888	Good fitness of the data
Q^2	>0.5	0.794	Excellent predictability
Model validity	>0.25	0.308	Valid model
Reproducibility	>0.5	0.959	Excellent reproducibility

2.4. *Bradford colorimetric protein assay*

Multiple protein assays are available, of which some measure amino acids and/or small peptides as well, *e.g.*, absorption spectrophotometry at 280 nm or the colorimetric assay with bicinchoninic acid [11–12]. However, for our purpose, quantification of BSA is required and not of its peptides or amino acids. Therefore, the Bradford assay was used, as this colorimetric assay determines only proteins with a molecular weight over 3000 Da. This method is based on an absorbance shift of the Coomassie Brilliant Blue G-250 dye which is converted from red to its blue form upon binding to the protein. It is this blue bound form that is detected at its absorption maximum at 595 nm [13]. In this study, the Bradford standard 3.1 mL assay protocol was followed as instructed by the supplier (Appendix A).

For the construction of the calibration curve, a standard solution series was made ranging between 0.25 mg/mL and 1.4 mg/mL. Also a blank solution was analyzed using 0.1 mL PBS. A linear regression model was used to calculate the BSA concentration in the examined samples.

3. Results and discussion

The results of this study are given in Table 1. Simple visual interpretation of the data already suggests that (i) not only the amount of ACN, but also FA percentage plays a major part in BSA precipitation, (ii) at low levels of ACN, the BSA loss is low and the variability of the results is high and (iii) varying the boiling time does not significantly influence the BSA loss.

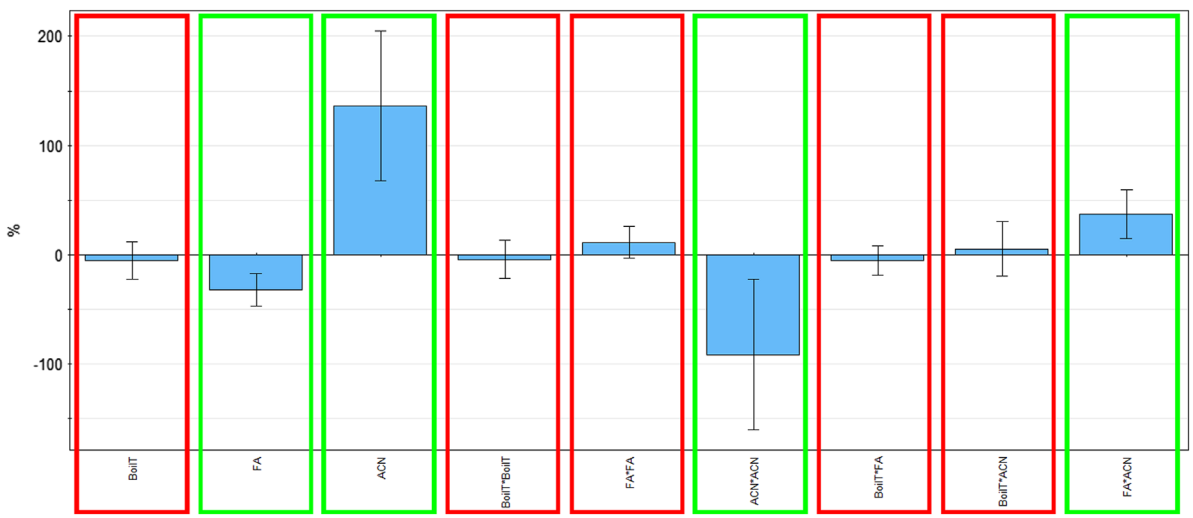

Fig. 1 Coefficient plot: different factors and interactions plotted in function of the percentage BSA loss. The red rectangles denote statistically insignificant factors and interactions ($\alpha = 0.01$), which were removed from the final model. The green boxes indicate the significant preserved factors and interactions.

Next, the BSA loss data were fitted using the most appropriate multiple linear regression (MLR) model. The coefficient plot in Fig. 1 represents the significance of all factors and interactions at $\alpha=0.01$. The green boxes indicate the significant factors and interactions, whereas the red boxes denote the insignificant ones (*i.e.*, their 99% confidence interval contains zero). The final model was composed by eliminating the statistically irrelevant factors and interactions and is described by: BSA loss (%)=–69.39 FA+42.68 ACN+0.80 FA × ACN–0.27 ACN^2–1601.55. The overall summary of fit of this model is presented in Table 2. When comparing the results obtained for the performance indicators (R^2, Q^2, model validity and reproducibility) with generally accepted reference values, this indicates a valid model with excellent predictability and reproducibility and a good fitness of the data [14].

If these results are applied on a real-life transdermal Franz diffusion cell (FDC) study, another critical quality attribute is defined: the lowest possible volume of ACN should be used in order to prevent too much sample dilution, leading to a decreased sensitivity in the assay method. Secondly, the sample preparation needs to be reproducible, requiring consistent BSA loss. Three user specifications are thus defined as analytical quality attributes: high BSA loss, low dilution factor and low variability. To determine the optimal working conditions, the Derringer desirability approach was applied, with the three desirability functions composed as presented in Fig. 2 [15–17]. The BSA loss was transformed to desirability d1 using an exponential function. Secondly, the robustness of BSA loss (d2) was modelled using a descending linear function. The residual standard deviations (RSDs) on the responses were calculated describing the variability at that ACN level; ideally, a low RSD is preferred. The desirability for the dilution factor (d3) was again described using a descending linear function, where a dilution factor (DF) of 50 was considered to be undesirable ($d=0.1$) and no dilution, *i.e.*, DF=1, was most desirable ($d=1$). These three functions were then used to transform our results into their corresponding d-values and subsequently, the global Derringer desirability (D) was calculated using the geometric mean $D=(d1 \times d2 \times d3)^{1/3}$.

The obtained d-values are given in Appendix B, and the global Derringer desirability D was modelled in function of the amount of ACN and FA, using multiple linear regression: $D=0.18$ ACN+0.074 FA–0.0016 FA^2–0.0012 ACN^2–0.00092 ACN × FA–5.82. Contour plots for the three individual quality attributes (expressed as d-values) as well as for the global desirability are shown in Fig. 3. Considering global D-values ≥ 0.9 as the desired design space, where the results are equally acceptable for our purposes, two optimal domains ($D=0.9$) can be defined in the contour plots, as visualized in Fig. 3 (red colored region) and derived from the D-equation given above: (1) FA > 4% and (2) FA < 2.5%. We have chosen the target point defined by 83% ACN and 1% FA as the optimal point within our experimental space. This target value is accepted to be the most robust condition, *i.e.*, where variations in percentage FA and ACN do not significantly influence the global D outcome. Moreover, lower FA percentages are also preferred for operational reasons, *i.e.* more appropriate for HPLC systems and prolonged column lifetime. While both factors are thus confounded with each other, in practice, these results also allow a more QC-operational approach as an individual tolerance: 83 ± 3% ACN and 1 ± 0.25% FA.

4. Conclusions

It goes without saying that a 'one size fits all' approach no longer has a place in modern analytical chemistry. Building-in quality should start early on, *i.e.*, at the method development stage. In contrast to the conventional HPLC-PDA equipment, sample preparation of samples containing BSA, *e.g.*, Franz diffusion cell solutions, demands special attention when novel fused core and/or UPLC-MS equipment is used. We suggested a QbD approach for sample pretreatment of BSA-containing samples. We have shown that not only the amount of ACN but also FA percentage plays a major part in BSA precipitation, while also considering the dilution effects and the variability in a global Derringer desirability

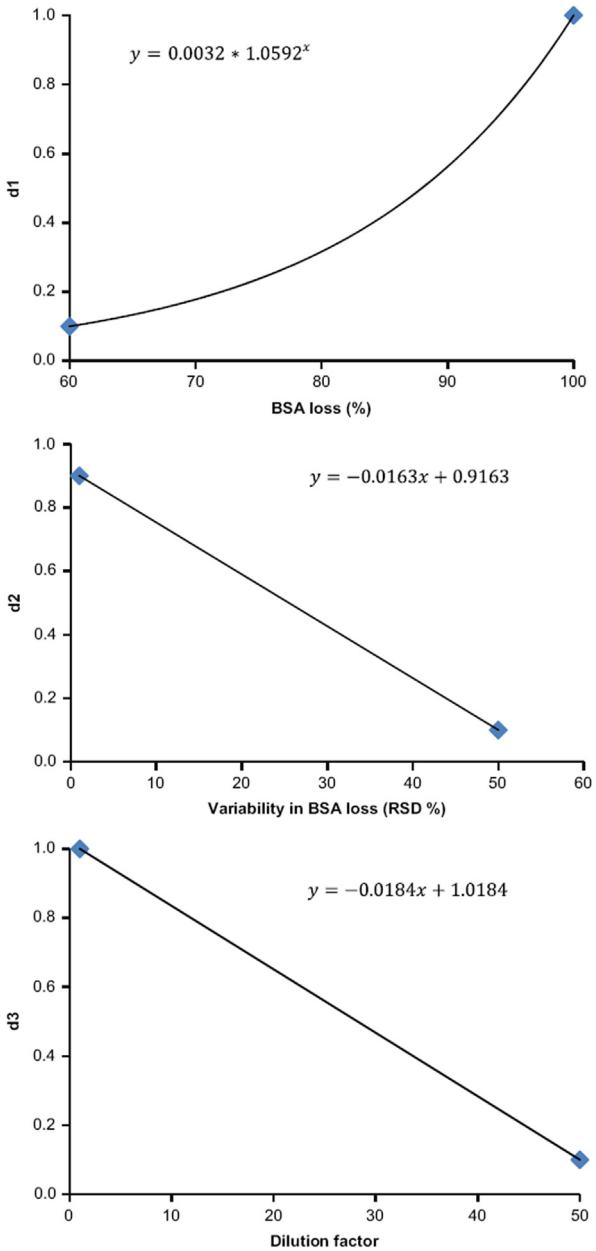

Fig. 2 Quality-by-design composed desirability functions with three identified critical quality attributes (BSA loss, variability in BSA loss and dilution factor) corresponding with our design space.

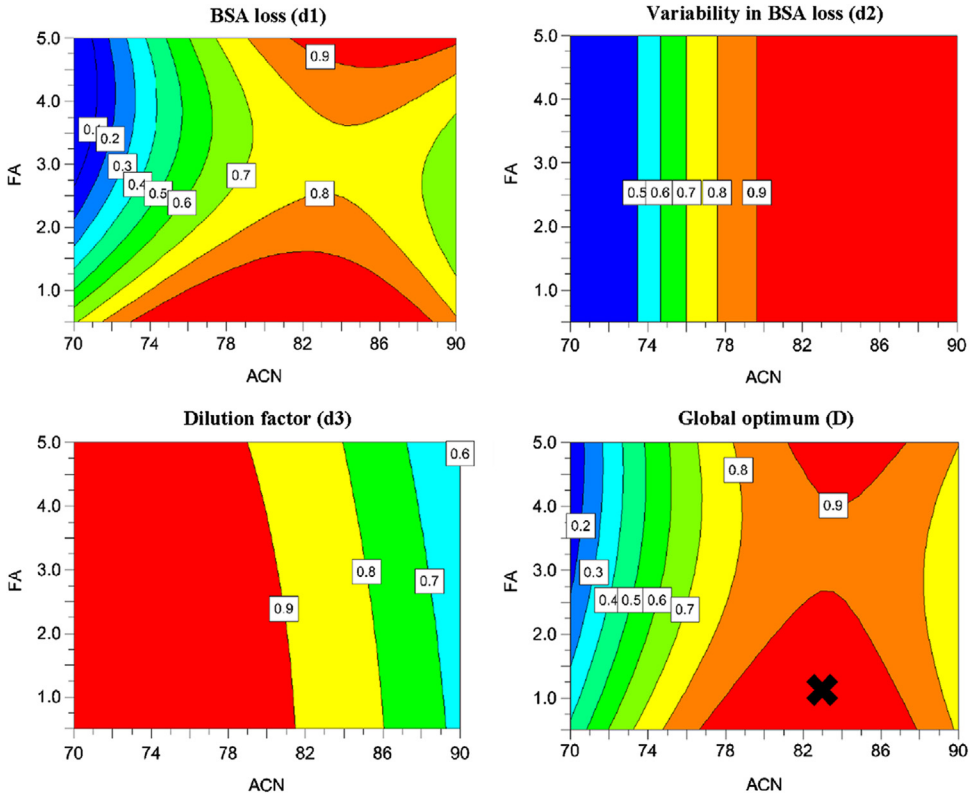

Fig. 3 Contour plots: d1 (BSA loss), d2 (variability in BSA loss), d3 (dilution factor) and *D* (global optimum) in function of the amount of ACN and FA.

(*D*) response. A well-defined design space was constructed, for which the target point was derived.

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Appendices A and B. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jpha.2014.06.001.

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